# A SPECTROSCOPIC ANALYSIS OF A HIGH FLUORESCENT MUTANT OF *CHLAMYDOMONAS REINHARDI*

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ABSTRACT Chloroplast fragments of a high fluorescent mutant of *Chlamydomonas reinhardi*, hfd 91, were compared against those of Acl$^+$, a low chlorophyll variant of the wild type. The chloroplast fragments of the mutant which have a high invariant fluorescence yield lacked photochemical activities associated with photosystem II (PSII) but retained normal photosystem I (PSI) activities. The mutant fragments also lacked the low temperature (−196°C) light-induced absorbance changes due to the photoreduction of C-550 and the photooxidation of cytochrome (cyt) *b*-559 which are PSII-mediated reactions. A fourth-derivative analysis of the absolute spectra of the chloroplast fragments at different stages of reduction (obtained with ferricyanide, ascorbate, and dithionite) showed both the oxidized and reduced forms of C-550 and the reduced forms of cyt *c*-553, *b*-559, and *b*-564 in wild-type fragments. The mutant fragments lacked C-550 and an ascorbate-reducible cyt *b*-559 but contained cyt *c*-553, a dithionite-reducible cyt *b*-559, and cyt *b*-564.

## INTRODUCTION

The use of mutant strains of algae has proven to be a powerful tool in elucidating electron transport systems of photosynthesis (1). Epel and Levine (2) described a method for obtaining mutant strains of the alga, *C. reinhardi*, which have lesions associated with PSII. (Also see Garnier [3] and Bennoun and Levine [4] for detecting mutants by their increased level of fluorescence.) In this paper we report on the spectroscopic analysis of a high fluorescent mutant of *C. reinhardi*. Difference spectra of the mutant and wild-type chloroplast fragments and a fourth-derivative analysis of the absolute spectra show that the mutant lacks C-550 and the ascorbate-reducible cyt *b*-559.

## MATERIALS AND METHODS

The low chlorophyll variant of the wild-type strain of *C. reinhardi*, Acl$^+$ (this strain will be referred to as the wild type in this paper because it has all of the normal photosynthetic

activities and components), and the high fluorescent mutant strain hfd 91 were grown in 300 ml of TAP medium (5) in 500 ml Erlenmeyer flasks at 30°C on a reciprocal shaker under cool white fluorescent lights. Chloroplast fragments were prepared from cells in the logarithmic phase of growth by a modification of the method of Levine and Gorman (6).

The chlorophyll content of the chloroplast fragments was determined by a modification (7) of the method of Mackinney (8). Rates of nicotinamide-adenine dinucleotide phosphate (NADP) and dichlorophenol indophenol (DCPIP) photoreduction were measured as previously described (2) under aerobic conditions with an Aminco-Chance double-beam spectrophotometer (American Instrument Co., Inc., Silver Spring, Md.). Fluorescence yield measurements were made with an instrument similar to that described by Yamashita and Butler (9). The mutant strain hfd 91 was obtained by a modification of the selection and screening method of Epel and Levine (2) and of Bennoun and Levine (4).

Absorption spectra were measured with a single-beam spectrophotometer on line with a PDP8/I computer described previously by Butler and Hopkins (10). The sample cell and Dewar flask for the measurement of spectra at liquid nitrogen temperature have been described previously (11). The phototube (EMI 9558C) was blocked with a 600 nm short bandpass interference filter (Optics Technology 717, Optics Technology, Inc., Palo Alto, Calif.) to reduce stray light from chlorophyll fluorescence. Samples were scanned four times and the spectral data were added together in the computer memory to improve the signal to noise ratio. Absolute spectra are presented as the difference between the spectrum of the sample and that of a frozen buffer blank. Fourth-derivative spectra were obtained from the computer using the method described by Butler and Hopkins (12) to enhance the signal to noise ratio in the fourth-derivative curves. The DX values used in the four differentiation steps were 2.0, 2.1, 2.2, and 2.5 nm. All spectral curves were plotted directly from the computer.

## RESULTS

Table I compares some of the photosynthetic electron transport reactions of chloroplast fragments from the mutant, hfd 91, and wild-type cells. The mutant chloroplast fragments show no Hill activity with either DCPIP or NADP as the electron acceptor; however, PSI activity as indicated by NADP photoreduction with the DCPIP-ascorbate couple as the electron donor is as great in the mutant as it is in the wild-type chloroplast fragments. The mutant chloroplast fragments, however, are not able to use PSII electron donors such as hydroquinone (HQ) plus ascorbate for the photoreduction of NADP.

The fluorescence yield properties of the chloroplast fragments from the mutant and wild-type cells are compared in Fig. 1. The fluorescence yield changes reflect the redox changes of $Q$, the fluorescence quencher and presumably the primary electron acceptor of PSII. The wild-type fragments show the typical fluorescence yield changes obtained in the absence of an electron acceptor. The fluorescence yield is low in the dark (or in weak measuring light) when $Q$ is oxidized and high in actinic light when $Q$ is reduced. The fluorescence yield is also high in the presence of dithionite (Dith) which reduces $Q$. In contrast, the fluorescence yield of the mutant chloroplast fragments is high in the measuring light and unchanged by actinic light. The high fluorescence yield is not lowered by ferricyanide (FeCN) and is increased only slightly by dithionite. The high, invariant, fluorescence yield suggests the loss or inactivation of either $Q$ or the PSII reaction center.

TABLE I

PHOTOSYNTHETIC ELECTRON TRANSPORT REACTIONS OF CHLOROPLAST FRAGMENTS FROM CELLS OF THE WILD TYPE ($Acl^+$) AND hfd 91 MUTANT STRAIN OF *C. REINHARDI**

| | Wild type | hfd 91 |
|---|---|---|
| NADP-Hill reaction | 148 | 0 |
| DCPIP-Hill reaction | 62 | 0 |
| DCPIP/Asc → NADP | 103 | 103 |
| HQ/Asc → NADP | — | 0 |

* All reactions were measured at 25°C in a 1 ml reaction mixture containing chloroplast fragments (equivalent to 12.5 μg chlorophyll) and, in micromoles: potassium phosphate buffer (pH 7.0), 10, KCl, 20; $MgCl_2$, 2.5. For the NADP-Hill reaction the reaction mixture also contained, in micromoles: *C. reinhardi* ferredoxin, 0.005; NADP, 0.5; and 50 M units ferredoxin-NADP reductase. For the DCPIP-Hill reaction 0.05 μmoles DCPIP were added. For the photoreduction of NADP using the DCPIP-ascorbate couple, to the reaction mixture was added, in micromoles: DCPIP, 0.05; ascorbate, 5; DCMU 0.02; *C. reinhardi* ferredoxin, 0.005; NADP, 0.05; and ferredoxin-NADP reductase, 50 M units. For the photoreduction of NADP using the HQ/Asc couple in addition to the reactants present for the NADP-Hill reaction were added, in micromoles: hydroquinone, 0.4; and ascorbate, 2.

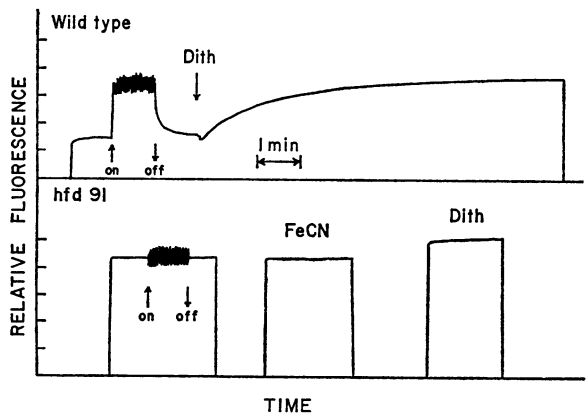


FIGURE 1 The fluorescence yield of wild-type ($Acl^+$) and mutant, hfd 91, chloroplast fragments (equivalent to 10 μg chlorophyll/ml) in 2 ml of the reaction mixture. The reaction mixture contained, in micromoles: potassium phosphate (pH 7.0), 10; KCl, 20; $MgCl_2$, 2.5; and where indicated potassium ferricyanide (FeCN), 1; dithionite (Dith), a few grains. Red actinic light (650 nm, $4 \times 10^4$ ergs $cm^{-2}$ $sec^{-1}$) turned on at upward arrow and off at downward arrow.

Knaff and Arnon (13) reported the presence of an electron transport component, C-550, which was reduced by PSII at liquid nitrogen temperature. They also showed that cyt *b*-559 was oxidized by PSII at these temperatures (14). Erixon and Butler

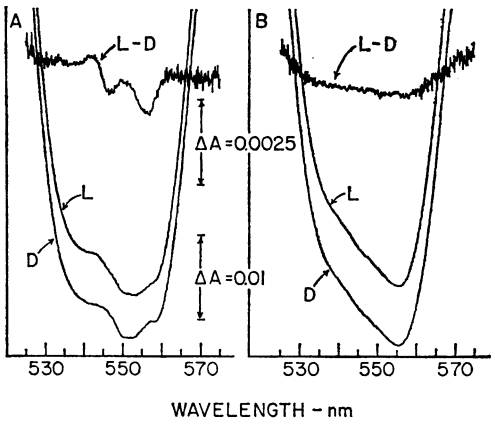


FIGURE 2

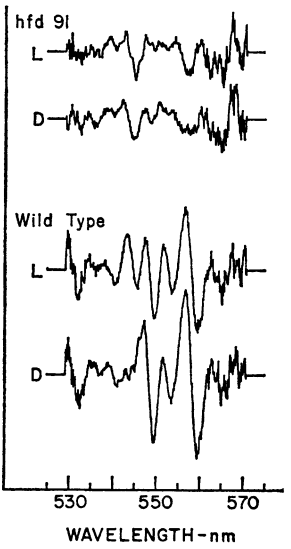


FIGURE 3

FIGURE 2 Absolute absorption spectra of chloroplast fragments equivalent to 100 μg chlorophyll in 0.5 ml at −196°C measured before (D) and after (L) irradiation with 645 nm light at −196°C, and light minus dark difference spectra (L − D). (A) Wild type (Acl+) and (B) mutant strain hfd 91. (Ordinate, absorbance, or Δ absorbance.)

FIGURE 3 Fourth-derivative curves of the absolute absorption spectra shown in Fig. 2.

(15) presented evidence, from redox titration data, that C-550 was isomorphic with *Q* and that the photooxidation of cyt *b*-559 and the photoreduction of C-550 at −196°C represented the electron-donating and electron-accepting ends of the same photoreaction, i.e., PSII. They also showed that the photoreduction of C-550 at −196°C is accompanied by an increase of absorbance at 543 nm as well as the bleaching at 547 nm, suggesting that the redox change of C-550 results in an absorption band shift to slightly shorter wavelength rather than the bleaching of the band.

Fig. 2 shows the absolute absorption spectra of fragments from the wild-type cells (A) and the mutant cells (B) before (D) and after (L) irradiation of the fragments with 645 nm light for 30 sec at −196°C. The light minus dark (L − D) difference spectra are also shown at higher sensitivity. The difference spectrum for the wild-type chloroplast fragments shows the same light-induced changes that were observed previously with spinach chloroplasts (15). The negative band at 557 nm is due to the photooxidation of cyt *b*-559 and the bleaching at 547 nm with the absorbance increase at 543 nm is due to the photoreduction of C-550. In the mutant strain no light-induced absorbance changes are observed in this spectral region. Previous work (15) with the high fluorescent mutant, No. 11, of *Scenedesmus* gave similar results. Thus, the absorbance data are consistent with the supposition that either *Q* or the reaction center is lacking or inactive in the mutant.

The absolute spectra of the mutant and wild-type fragments show differences, particularly in the region around 545 nm, but these differences are difficult to define

because they involve overlapping absorption bands and steep sides due to the bulk chlorophylls and carotenes. The use of higher derivative analysis to resolve and obtain information from complex spectra was recently described (10, 12). The fourth derivative of a symmetrical absorption band shows a maximum at the same position as the absorption maximum but spectral resolution may be enhanced markedly because the bandwidth of the fourth-derivative band is much narrower than the original band. Also, the higher derivative analysis can be made highly selective for particular bands by matching the differentiating interval to the width of specific bands. This arises because the amplitude of the *n*th derivative of a band is proportional to $w^{-n}$, where $w$ is the half-width. Thus, narrow bands are discriminated for in favor of broader bands (e.g., the fourth derivative of a 10-nm-wide band will be 16 times greater than that for a 20-nm-wide band of equal magnitude) provided the differentiating interval is not too large to resolve the narrow band. On the other hand broad bands can be selected for over narrow bands by using wider differentiating intervals which do not resolve the narrow bands but increase the derivative signal according to $(\Delta\lambda)^n$, where $\Delta\lambda$ is the differentiating interval. With these techniques the absorption bands due to C-550 and the various cytochromes can be resolved from the absolute spectra.

Fig. 3 shows the computer-generated fourth-derivative curves of the absolute spectra presented in Fig. 2. The fourth-derivative spectra of the chloroplast fragments from wild-type cells show bands at 547 and 552 nm due to the split $\alpha$ band of reduced cyt *c*-553 at −196°C and a band at 557 nm due to reduced cyt *b*-559. The derivative band of the oxidized C-550 in curve D of the wild-type spectra is hidden under the 547 band. (The oxidized C-550 band will be shown without interference from other bands in the following figure.) On irradiation the absorption band of C-550 shifts to shorter wavelength and the fourth derivative band appears at 543 nm (curve L of wild type). The derivative band at 557 nm is not quite as large after irradiation due to photooxidation of a part of the cyt *b*-559. The corresponding fourth-derivative spectra of the mutant chloroplast fragments show no distinguishable bands thus establishing the absence of C-550; however, the absence of the cytochrome bands could be due to their being in the oxidized state.

Fig. 4 compares fourth-derivative spectra of wild-type and mutant chloroplasts, A and B, respectively, after treatment with ferricyanide (curve 1, FeCN), ascorbate (curve 2, Asc), and dithionite (curve 3, Dith). The oxidized wild-type chloroplast fragments (curve 1 of Fig. 4 A) show only the fourth-derivative band at 546 nm due to the oxidized C-550. No comparable band is observed in the mutant fragments with ferricyanide (curve 1 of Fig. 4B). On reduction of the wild-type fragments with ascorbate (curve 2 of Fig. 4 A) cyt *c*-553 with the split $\alpha$ band at 547 and 552 and cyt *b*-559 with the band at 557 nm appear. In the mutant (curve 2 of Fig. 4 B) reduction of cyt *c*-553 occurs but not cyt *b*-559. Addition of dithionite to the wild-type fragments (curve 3 of Fig. 4 A) reduces C-550 as shown by the absorption band shift to 543 nm, the band for cyt *b*-559 increases somewhat as there appears

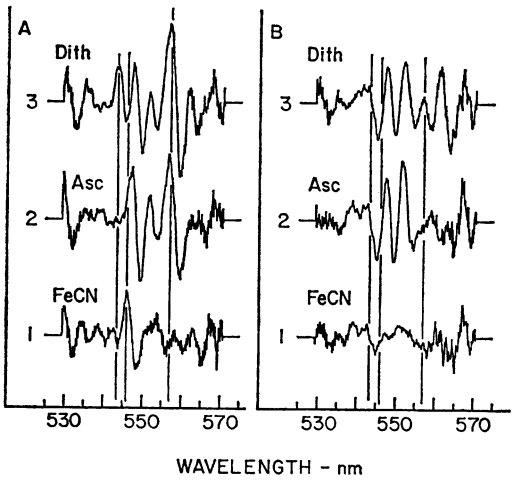


FIGURE 4

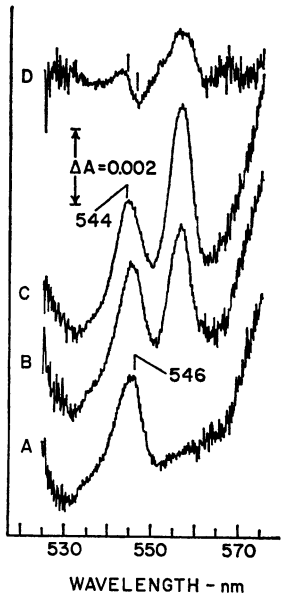


FIGURE 5

FIGURE 4 Fourth-derivative absorption spectra of chloroplast fragments (equivalent to 100 μg chlorophyll in 0.5 ml) of (A) wild type ($Acl^+$) and (B) mutant hfd 91 at −196°C in the presence of (1) 2 μmoles FeCN, (2) 20 μmoles Asc, (3) a few grains of Dith. Wavelength lines are placed at 543.5, 546, and 557 nm.

FIGURE 5 Low temperature difference absorption spectra of wild-type vs. hfd 91 chloroplast fragments (100 μg in 0.5 ml) in the presence of (A), 2 μmoles ferricyanide, (B) 20 μmoles ascorbate, (C) a few grains dithionite. Curve D, difference between curves C and B. (Ordinate, absorbance, or Δ absorbance.)

to be some cyt *b*-559 which is dithionite but not ascorbate reducible and the band for cyt *b*-564 (analogous to cyt $b_6$) appears at 562 nm. The mutant chloroplast fragments (curve 3 of Fig. 4 B) show the dithionite-reducible *b*-559 and the cyt *b*-564.

The absorption bands for the components absent from the mutant chloroplast fragments can be seen in difference spectra between the wild type and the mutant at equal chlorophyll concentrations and oxidation states (Fig. 5). The difference spectrum with ferricyanide in both samples, curve A, shows the oxidized band of C-550 which is present in the wild type but absent from the mutant. The difference spectrum in the presence of ascorbate, curve B, shows the oxidized band of C-550 and the ascorbate-reducible cyt *b*-559 which are absent from the mutant. The difference spectrum with dithionite, curve C, shows the reduced band of C-550 and somewhat more cyt *b*-559 than was observed with ascorbate. Thus, the wild type contains more of the dithionite-reducible (nonascorbate-reducible) cyt *b*-559 than the mutant. Curve D, the difference spectrum between curves C and B, shows the band shift due to reduction of C-550 and the greater amount of the cyt *b*-559 which is dithionite reducible but not ascorbate reducible in the wild-type chloroplasts.

## DISCUSSION

The spectra clearly show that the hfd 91 mutant strain lacks C-550 which has been identified as the primary electron acceptor of PSII. The absence of the absorbance changes in the light minus dark difference spectra would not distinguish between absence and inactivation. The absolute absorption bands can be resolved by the fourth-derivative analysis and these spectra, as well as the wild-type minus mutant difference spectra, establish the presence or absence of the light-absorbing components.

Six other high fluorescent mutants of *C. reinhardi* have been subjected to the same spectroscopic analysis including Ac141, a mutant strain previously reported by Levine et al. (16) to be lacking cyt *b*-559. All are lacking C-550 and the ascorbate-reducible cyt *b*-559. The cyt *b*-559 which is photooxidized by PSII at −196°C is a high potential cyt *b* with a midpoint potential higher than +400 mv (17). In order to be involved in the photoreaction at −196°C the cytochrome must be in close physical association with PSII. Boardman and Anderson (18) found that cyt *b*-559 was associated with the PSII particles of spinach chloroplasts and Vernon et al. (19) reported that cyt *b*-559 was present in particles they considered to be the reaction centers of PSII. Where this cyt *b*-559 functions in the electron transport system is not clear.

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## REFERENCES

1. LEVINE, R. P. 1969. *Annu. Rev. Plant Physiol.* **20**:523.
2. EPEL, B. L., and R. P. LEVINE. 1971. *Biochim. Biophys. Acta.* **226**:154.
3. GARNIER, J. 1967. *C.R. Hebd. Seances Acad. Sci. Ser. D. Sci. Nat. (Paris).* **265**:874.
4. BENNOUN, P., and R. P. LEVINE. 1967. *Plant Physiol.* **42**:1284.
5. GORMAN, D. S., and R. P. LEVINE. 1965. *Proc. Natl. Acad. Sci. U.S.A.* **54**:1665.
6. LEVINE, R. P., and D. S. GORMAN. 1966. *Plant Physiol.* **43**:1293.
7. ARNON, D. I. 1949. *Plant Physiol.* **24**:1.
8. MACKINNEY, J. 1941. *J. Biol. Chem.* **140**:315.
9. YAMASHITA, T., and W. L. BUTLER. 1968. *Plant Physiol.* **43**:1978.
10. BUTLER, W. L., and D. W. HOPKINS. 1970. *Photochem. Photobiol.* **12**:439.
11. NORRIS, K. H., and W. L. BUTLER. 1961. *IRE (Inst. Radio Eng.) Trans. Bio-Med. Electron.* **8**:153.
12. BUTLER, W. L., and D. W. HOPKINS. 1970. *Photochem. Photobiol.* **12**:451.
13. KNAFF, D. B., and D. I. ARNON. 1969. *Proc. Natl. Acad. Sci. U.S.A.* **63**:963.

14. KNAFF, D. B., and D. I. ARNON. 1969. *Proc. Natl. Acad. Sci. U.S.A.* **63**:956.
15. ERIXON, K., and W. L. BUTLER. 1971. *Biochim. Biophys. Acta.* **234**:381.
16. LEVINE, R. P., D. S. GORMAN, M. AVRON, and W. L. BUTLER. 1966. *Brookhaven Symp. Biol.* **19**:143.
17. ERIXON, K., R. LOZIER, and W. L. BUTLER. 1972. *Biochim. Biophys. Acta.* **267**:375.
18. BOARDMAN, N. K., and J. M. ANDERSON. 1967. *Biochim. Biophys. Acta.* **143**:187.
19. VERNON, L. P., E. R. SHAW, T. OGAWA, and D. RAVEED. 1971. *Photochem. Photobiol.* **14**:343.